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Hepatocyte growth factor induces branching tubulogenesis in MDCK cells by modulating the activin-follistatin system

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Hepatocyte growth factor induces branching tubulogenesis in MDCK cells by modulating the activin-follistatin system.

Background. The activin-follistatin system is expressed in tubular cells of the kidney. The present study was conducted to examine the role of the activin-follistatin system in tubulogenesis using Madin-Darby canine kidney (MDCK) cells as a model system.

Methods. Tubulogenesis was assessed using MDCK cells cultured in collagen gel. The effect of recombinant human activin A on tubulogenesis was examined. Blockade of the action of endogenous activin was achieved by either adding follistatin or transfection of dominant-negative mutant of the type II activin receptor. The production of activin A was examined by Northern blotting, in situ hybridization, and Western blotting.

Results. MDCK cells expressed mRNA for the β_A subunit of activin. These cells formed spherical cysts when cultured in collagen gel. Hepatocyte growth factor (HGF) added to the spherical cysts induced branching tubulogenesis. When activin A was added together with HGF, activin A blocked the branching tubulogenesis induced by HGF, and the activin-treated cells were scattered. Conversely, follistatin, an antagonist of activin A, induced branching tubulogenesis qualitatively similar to that induced by HGF. Adenovirus vector-mediated transfer of the gene encoding truncated type II activin receptor, which acts as a dominant negative mutant, also induced branching tubulogenesis. Finally, HGF markedly inhibited the production of activin A in MDCK cells cultured in collagen gel.

Conclusion. Activin A produced in MDCK cells tonically inhibits branching tubulogenesis, and HGF induced branching tubulogenesis mainly by blocking the production of activin A.

Interaction between epithelial and mesenchymal cells is essential during mammalian development. In the kidney, mesenchymal cells produce a factor(s) that is necessary for branching morphogenesis of the renal epithelia [1]. A factor(s) secreted from mesenchyme thus acts as morphogens during kidney development.

Hepatocyte growth factor (HGF) is a multifunctional factor that modulates cell growth, differentiation, motility, and morphogenesis [2, 3]. The effect of HGF as a morphogen was studied in Madin-Darby canine kidney (MDCK) cells, a model cell system for the formation of renal tubules [4, 5]. These cells have the functional properties of renal distal tubules and form tubule-like structures in vitro. When cultured in a collagen gel, these cells form spherical cysts [4]. HGF added to the spherical cysts induces drastic changes in morphology. Thus, HGF-treated MDCK cells form branching epithelial tubular structures that resemble renal tubules [5]. Since HGF is synthesized in mesenchymes during development, HGF produced from mesenchymal cells is now thought to play a critical role as a morphogen in the embryonic development of tubular kidney structures [6].

Activins are multifunctional cytokines structurally related to transforming growth factor-β (TGF-β). Like other members of the TGF-β supergene family, activins modulate cell proliferation, induce differentiation, and are involved in organogenesis and tissue remodeling [7]. Activins are dimeric proteins, and subunits of activin are expressed in various organs [8]. Two structurally different proteins modulate the action of activins. In gonadal tissue and the pituitary, the action of activins is blocked by inhibin, another member of the TGF-β supergene family [9]. Another important modulator of activin action is follistatin [10, 11]. This protein specifically binds to activins and related ligands with high affinity and blocks their action [12, 13]. In general, activins exert their actions as autocrine or paracrine factors [11]. Follistatin, an activin antagonist, is synthesized in the target cells of activins and remains in the extracellular matrix. Furthermore, the production of follistatin is regulated by activins. Hence, activin and follistatin modulate cellular function in a complex manner.

Key words: kidney, development, scatter factor, cell differentiation, recombinant human activin A, renal tubules, organogenesis.

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Among activins, activin A is expressed in the kidney [14]. Follistatin is also abundantly expressed in this organ [15]. Given that members of the TGF-β supergene family are involved in the development of the kidney [16, 17], it is possible that activin A also modulates organogenesis of the kidney as an autocrine factor. In this regard, activin A has been shown to block branching morphogenesis of the renal tubule [18]. To determine the role of activin A in the tubulogenesis, we studied the involvement of the activin-follistatin system in tubulogenesis using MDCK cells as a model system. The results indicate that HGF induces tubulogenesis by modulating the activin-follistatin system.

METHODS

Materials

Recombinant human activin A and follistatin were provided by Dr. Y. Eto of the Central Research Laboratory (Ajinomoto Inc., Kawasaki, Japan). Recombinant human HGF was obtained from Funakoshi (Tokyo, Japan). Recombinant human TGF-β1 was obtained from Wako Pure Chemicals (Osaka, Japan). Recombinant human insulin-like growth factor-I (IGF-I) was a gift from Fujisawa Pharmaceutical Co. Ltd. (Osaka, Japan). Epidermal growth factor (EGF) was purchased from Collaborative Research (Lexington, MA, USA). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from GIBCO BRL (Grand Island, NY, USA). [^{3}H]Thymidine and [^{32}P]deoxycytidine triphosphate were purchased from DuPont-New England Nuclear (Boston, MA, USA).

Cell line and collagen gel culture

Madin-Darby canine kidney cells obtained from the American Type Culture Collection were maintained in a "complete medium" comprised of DMEM supplemented with 5% FBS and antibiotics (penicillin, streptomycin) under humidified conditions of 95% air and 5% CO_2 at 37°C. The culture medium was changed every three to four days. To obtain quiescent cells, cells were incubated in a serum-free medium for 72 hours.

For the collagen gel culture, cells were suspended at 5×10^3 cells/mL in a neutralized collagen solution (Koken, Tokyo, Japan), dispensed into 24-well plates, and incubated at 37°C. After the collagen solution had gelled, the complete culture medium with the indicated agents was added and renewed every two to three days. The cultures were photographed at the indicated times under phase contrast using a Nikon Diaphot TMD inverted microscope (Tokyo, Japan).

Measurement of DNA synthesis

DNA synthesis was assessed by measuring [^{3}H]thymidine incorporation into trichloroacetic acid-precipitable materials. Serum-starved cells cultured in a 24-well plate were incubated in complete medium with the indicated agents for 16 hours. Then the cells were pulse labeled with 1 μCi/mL [^{3}H]thymidine for an additional four hours. [^{3}H]Thymidine incorporation was measured as described by McNiel, McKenna, and Taylor [19].

RNA extraction and Northern blot analysis

Total RNA (30 μg), extracted from MDCK cells using TRIzol Reagent (GIBCO BRL), was denatured on a 1.2% agarose gel containing 2.2 mol/L formaldehyde and blotted onto a nylon membrane (Hybond-N+; Amersham Japan, Tokyo, Japan) via capillary blotting in a 20× saline-sodium citrate buffer (SSC). The membrane was prehybridized for two hours at 42°C in a hydridization buffer comprised of 50% (vol/vol) formamide, 5 × standard saline phosphate-ethylenediaminetetraacetic acid (SSPE), 5 × Denhart's solution, 100 mg/mL denatured salmon sperm DNA and 0.5% sodium dodecyl sulfate (SDS), and then hybridized for 16 to 24 hours at 42°C with the following cDNA fragment, labeled with [^{32}P] by random priming using a DNA labeling kit (-dCTP; Pharmacia, Uppsala, Sweden): cDNA for rat follistatin (provided by Dr. S. Shimasaki of the Salk Institute, La Jolla, CA, USA), cDNA for the human β_A subunit (provided by Dr. Y. Eto), and cDNA for human GAPDH (Clonetech Laboratories, Inc., Palo Alto, CA, USA). The membrane was washed four times in 0.1 × SSC and 0.1% SDS at room temperature for 5 minutes and then at 55°C for 30 minutes. The membrane was subjected to autoradiography, analyzed using a Fujix BAS 2000, and photographed with a Fujix Pictography 3000 (Fuji Photo Film, Tokyo, Japan). To quantitate the β_A mRNA content in each lane, the intensity of the autoradiographic signal for either β_A or GAPDH was quantitated and expressed as an arbitrary density unit. The ratio of the integrated signal of β_A/GAPDH was determined for each sample. To isolate RNA from collagen gel cultures, MDCK cells cultured in collagen gel for four days to form spherical cysts were incubated with HGF for indicated times. Then a small piece of collagen gel was manually removed and digested by incubation with type I collagenase (Worthington Biochemical Corp., Lakewood, NJ, USA) at 37°C for 30 minutes. The released cells were collected by centrifugation and resuspended in TRIzol Reagent. Northern blotting was performed as described previously in this article.

Western blot analysis

Madin-Darby canine kidney cells were washed three times with phosphate-buffered saline (PBS), suspended in Laemmli buffer, and heated to 100°C for 10 minutes. After centrifugation, supernatant was collected, and the protein concentration was determined by using a protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA).

Twenty micrograms of protein from each sample were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing condition and transferred to a polyvinylidine difluoride (PVDF) membrane (Nihon Millipore Ltd., Yonezawa, Japan) by electroblotting. To reduce nonspecific antibody binding, the membrane was blocked with 5% bovine serum albumin (BSA), 0.1% NaN_3 dissolved in Tris-saline (TS) for one hour at 37°C, then incubated overnight with polyclonal anti-human activin A antibody [36], and washed with Tris-PBS (PBST). After incubation with peroxidase-labeled anti-rabbit IgG antibody for one hour at room temperature, the membrane was washed with PBST and analyzed by exposure to x-ray film using ECL™ Western blotting detection reagent (Amersham Life Science).

In situ hybridization

The cRNA prove was transcribed from a pMEP4 vector containing a 1.9 kb Xho I fragment derived from human β_A subunit cDNA (provided by Dr. Y. Eto). Linearized plasmids were used for in vitro transcription of digoxigenin-11-UTP–labeled antisense and sense riboprobe with T3 and T7 RNA polymerase, respectively, according to the manufacturer's instructions (Boehringer-Mannheim GmbH, Mannheim, Germany). MDCK cells, which formed spherical cysts or branching tubules in collagen gel, were fixed in 4% paraformaldehyde (PFA) in PBS at 4°C and embedded in paraffin. Four micrometer sections were mounted on poly-L-lysine-coated slides, deparaffinized, and dehydrated in a routine manner. After digestion with 5 μg/mL proteinase K at room temperature for 30 minutes, they were fixed in 0.4% PFA at 4°C for 20 minutes and incubated at 50°C overnight with the hydridization buffer containing 1 μg/mL DIG-labeled cRNA. The composition of the buffer was 50% formamide, 10 mmol/L Tris-HCl (pH 7.5), 600 mmol/L NaCl, 1 mmol/L EDTA, 0.25% SDS, 1 × Denhard't solution, 200 μg/mL yeast tRNA, and 10% dextran sulfate. After hydridization, sections were washed in 2 × SSC/50% formamide at 58°C for 30 minutes, incubated in 1 μg/mL RNase A solution at 37°C for 30 minutes, and washed once in 2 × SSC and twice 0.2 × SSC at 50°C for 20 minutes each time. Sections were then incubated in a 1:500 diluted solution of polyclonal sheep anti-DIG Fab antibody conjugated with alkaline phosphatase before washing and detection of the label with nitroblue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolylphosphate (BCIP).

Construction of a replication-deficient recombinant adenovirus vector

The recombination adenovirus AdextARII, carrying truncated type II activin receptor (tARII) cDNA as described previously [20], was generated by homologous recombination between the expression cosmid cassette and the parental virus genome as described by Miyake et al [21]. Briefly, the expression cosmid cassette was constructed by inserting the expression unit into the Swa I site of pAdex1cw (provided by I. Saito, University of Tokyo, Tokyo, Japan). The expression unit comprised a cytomegalovirus (CMV) promoter, tARII cDNA, and a poly A sequence. The expression cosmid cassette and adenovirus DNA-terminal protein (DNA-TPC) were cotransfected into 293 cells using a DOTAP Liposomal Transfection Reagent (Boehringer Mannheim). The desired recombinant adenovirus was amplified in 293 cells, purified using the CsCl density centrifugation method, and stored at −80°C. The titer of vital stocks was determined by plaque formation assay using 293 cells. The recombinant adenovirus AdexLacZ, carrying the lacZ gene that codes for the *Escherichia coli* β-galactosidase, was provided by Dr. T. Takeuchi (Gunma University, Maebashi, Japan) and was used as a control for confirming successful transfection.

In vitro transfection

Spherical cysts obtained after four days of culture in collagen gel were used for in vitro adenovirus vector infection. Cysts in the collagen gel were incubated in various concentrations of viral solution for 24 hours at 37°C. The viral solution was prepared by diluting viral stocks with the complete medium. The collagen gel was washed with a serum-free medium and then cultured in the complete medium for the indicated periods.

Detection of β-galactosidase activity

The collagen gels were fixed with 0.25% glutaraldehyde for five minutes at 4°C and then rinsed with PBS three times and incubated for three hours in a staining solution at 37°C. The staining solution was comprised of 1 mg/mL of 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal), 1 mmol/L $MgCl_2$, 3 mmol/L $K_3Fe(CN)_6$, 3 mmol/L $K_4Fe(CN)_6$, 0.1% Triton X-100, and 10 mmol/L KCl, in a 100 mmol/L sodium phosphate buffer (pH 7.5). β-Galactosidase expression was detected as the development of blue pigmentation caused by the enzymatic cleavage of X-gal [22].

Processing for light microscopy

Collagen gels were fixed with 4% formaldehyde in PBS, removed from the cultured dishes, dehydrated in graded concentrations of ethanol, and embedded in paraffin blocks according to standard procedures. Four micrometer sections were cut and stained with hematoxylin and eosin.

Processing for electron microscopy

Collagen gel cultures were fixed in situ overnight with solution containing 2.5% glutaraldehyde, 2% PFA in 0.1 mol/L sodium phosphate buffer (PB), pH 7.4. Then the

collagen gels were removed from the culture dishes and cut into an approximately 3 × 3 mm fragments. These were postfixed in 1% osmium tetroxide in 0.1 mol/L PB for one hour, dehyrated with ethanol, and embedded in Epon. Thin sections were cut, stained with uranyl acetate and lead citrate, and examined with a JEM1010 electron microscope (JEOL, Tokyo, Japan).

Semiquantitative analysis of tubular structure formation

Madin-Darby canine kidney cells suspended in collagen gels were incubated with HGF in the presence or absence of various concentrations of activin A. After seven days, 30 colonies per experimental condition were randomly selected and photographed under a phase-contrast microscope, and then the percentage of tubular structures was determined in each of three separate experiments.

To examine the tubulogenic activities of HGF and follistatin in MDCK cells cultured in collagen gel, morphological change after 10 days culture with indicated factors was observed and divided into three groups: (*1*) smooth cysts, (*2*) irregularly shaped cysts (which had no apparent tubules, but their cyst wall was not smooth), and (*3*) branching tubules. Quantitation of branching was also performed by counting all identifiable branch points in each colony. The mean values for each condition were compared.

RESULTS

Effect of recombinant activin A and TGF-β on MDCK cell proliferation

Activin A affects the growth of many types of cells [11]. We first measured the effect of activin A on DNA synthesis in MDCK cells cultured in a monolayer culture. When serum-starved cells were incubated in a complete medium containing 5% FBS, DNA synthesis began to increase after a 12-hour interval and markedly increased thereafter. Activin A did not have any effect on FBS-induced DNA synthesis in MDCK cells, even at concentrations of up to 20 nmol/L and above (data not shown). In contrast, TGF-β markedly inhibited DNA synthesis in MDCK cells at a concentration of 100 pmol/L. Morphologically, there was no change in cells treated with activin A.

Expression of mRNA for the β_A subunit in MDCK cells

We then examined the expression of mRNA for the β_A subunit of activin in MDCK cells. Figure 1 shows the changes in mRNA for the β_A subunit in serum-starved cells after the addition of FBS. mRNA expression of the β_A subunit was detected on quiescent cells and increased significantly after 3 hours and returned to the basal level

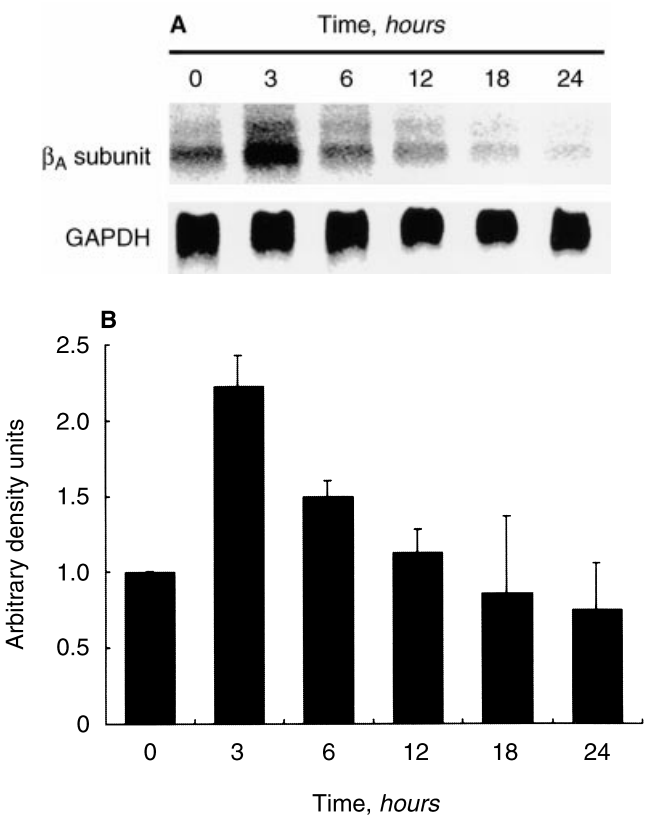
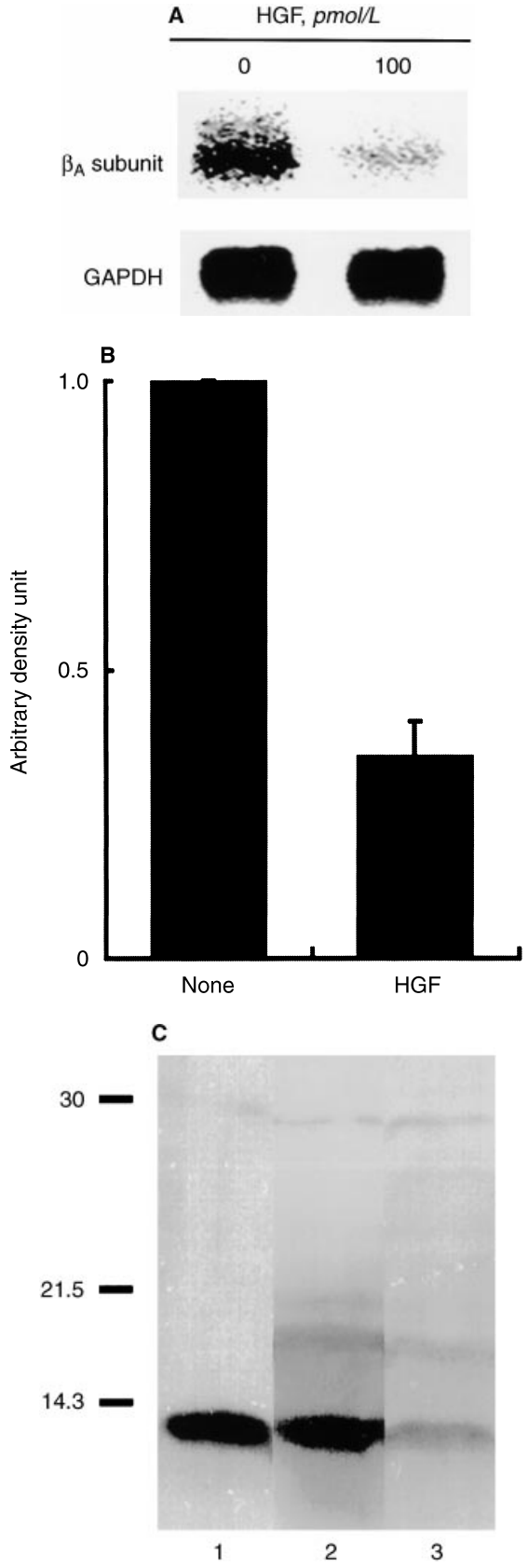


Fig. 1. Expression of mRNA for the β_A subunit of activin in MDCK cells in a monolayer culture. (*A*) MDCK cells cultured in a 100 mm dish were incubated for 72 hours in a serum-free medium. Cells were then incubated with 5% FBS for the indicated time, and mRNA expression of the β_A subunit and GAPDH was measured with Northern blotting. Results are representative of three experiments. (*B*) Quantitation of β_A/GAPDH mRNA in MDCK cells incubated with 5% FBS for the indicated time. Values are the mean ± SE of three experiments and are expressed as arbitrary density units.

within 12 hours of the addition of FBS. We then examined whether the mRNA expression of the β_A subunit was affected by growth factors involved in kidney development. Figure 2A and B show the effect of HGF on the serum-induced elevation of the mRNA expression for the β_A subunit. Serum-starved MDCK cells were incubated for three hours with 5% FBS in the presence or absence of HGF. The addition of HGF markedly decreased the mRNA level of the β_A subunit. To confirm the effect of HGF on the production of activin A in MDCK cells, we performed Western blotting using anti-human activin A antibody. As shown in Figure 2C, a 12.5 kD band was detected under reducing condition in the lysate of serum-treated MDCK cells, which was reduced in HGF-treated MDCK cells. We examined the effect of other growth factors, including epidermal growth factor (EGF, 1 to 10 nmol/L), insulin-like growth factor (IGF-I, 1 to 10 nmol/L), and TGF-β (10 to 100 pmol/L) on the expression of the β_A subunit mRNA. These factors did not affect the expression of β_A subunit.



Inhibition of HGF-induced branching morphogenesis by activin A

To investigate whether the morphogenic behavior of MDCK cells was influenced by activin A, we first examined the effect of activin A on MDCK cells cultured in collagen gel. MDCK cells in collagen gel were cultured in the complete medium in the presence or absence of activin A. After seven days, MDCK cells formed spherical cysts in the absence of activin A (Fig. 3A). In the presence of activin A, MDCK cells also formed spherical cysts, the same as in the control cultures (data not shown). Activin A alone did not have any tubulogenic activity for MDCK cells. Next we examined the effect of activin A on tubule formation induced by HGF. MDCK cells in collagen gel were cultured in the complete medium containing HGF in the presence or absence of activin A. In the presence of HGF, MDCK cells formed tubule-like structures, which had slit-like translucent spaces, within 10 to 15 days (Fig. 3B, C). In the presence of HGF and activin A, MDCK cells did not form tubule-like structures. Instead, these cells scattered and the spherical cysts disappeared (Fig. 3D). This inhibitory effect of activin A on branching tubulogenesis was observed at 4 nmol/L and above (Fig. 4).

Induction of tubule formation by follistatin

To assess the role of endogenously produced activin A in the tubulogenesis of MDCK cells, we examined the effect of follistatin on MDCK cells in collagen gel. Follistatin is an activin-binding protein and blocks the effect of activin A and related factors [12, 13]. MDCK cells in collagen gel were cultured in the complete medium in the presence or absence of follistatin. Surprisingly, follistatin induced the extension of processes radially from the wall of the cysts into the surrounding collagen matrix after six days (Fig. 5A). The processes then formed many branching tubule-like structures, which had slit-like translucent spaces, within 10 to 15 days (Fig. 5B–D). Thin-section electron microscopy of MDCK cells treated with follistatin showed polarized tubular formation with smooth basal surface in contact with the collagen gel and microvilli-rich apical surface facing the lumen, thus demonstrating the tubular nature of branching cords

Fig. 2. Effect of hepatocyte growth factor (HGF) on the production of activin A. (*A*) Serum-starved cells were incubated with 5% FBS in the presence or absence of 100 pmol/L HGF. mRNA expression of the β_A subunit and GAPDH was measured with Northern blotting. Results are representative of three experiments. (*B*) Quantitation of β_A/GAPDH mRNA in MDCK cells treated with or without HGF. Values are the mean ± SE of three experiments and are expressed as arbitrary density units. (*C*) Immunoblot analysis of MDCK cells treated with (3) or without (2) HGF for 24 hours using anti-human activin A antibody. (1) Recombinant human activin A (100 ng).

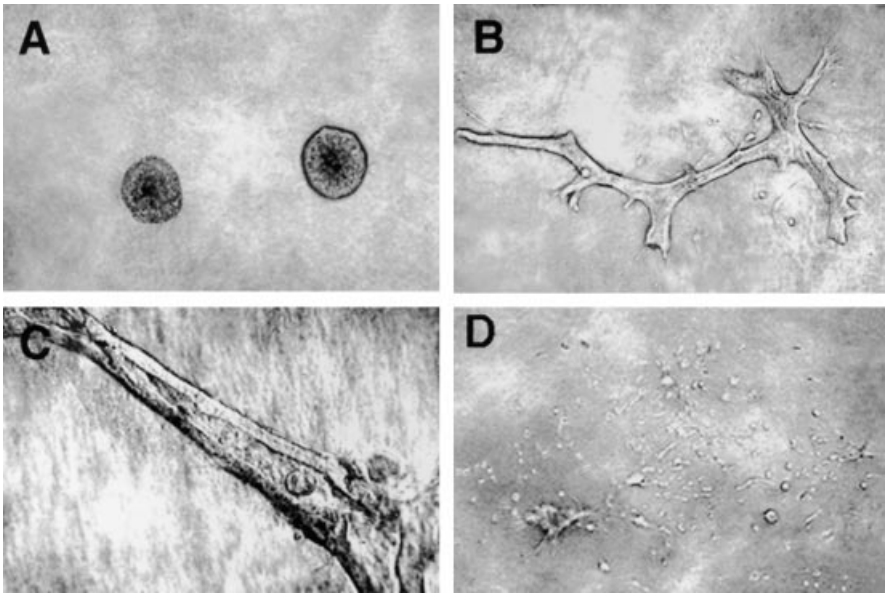


Fig. 3. Effect of HGF and activin A on the morphology of MDCK cells cultured in collagen gel. MDCK cells were cultured in collagen gel as described in the **Methods** section. Cells were incubated in the complete medium in the presence or absence of various agents. (*A*) None for seven days (×100). (*B*) A total of 250 pmol/L HGF for seven days (×100); (*C*) 250 pmol/L HGF for 10 days (×400); (*D*) 250 pmol/L HGF and 10 nmol/L activin A for seven days (×100).

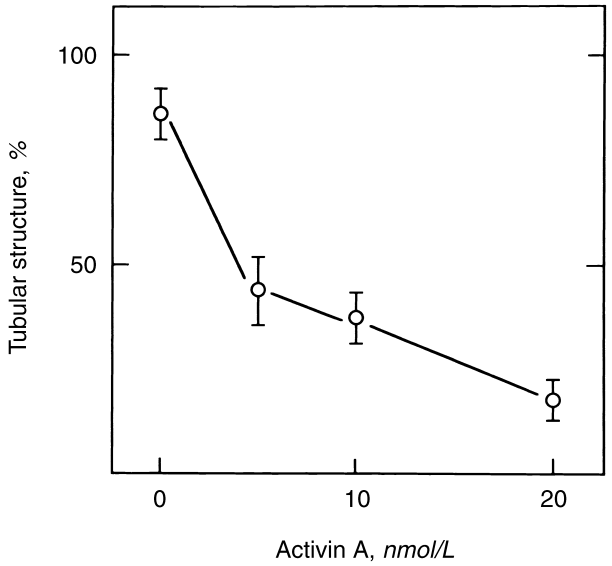


Fig. 4. Dose–response relationship for activin A-induced inhibition of tubulogenesis. MDCK cells were cultured in collagen gel in the presence of 250 pmol/L HGF and various doses of activin A for seven days. The number of tubular structures was then counted. Values are the mean ± SE of three experiments.

(Fig. 6). This inductive effect of follistatin was observed at concentrations of 1 nmol/L and above. We then examined the effect of follistatin on tubule formation induced by HGF. In the presence of HGF and follistatin, MDCK cells formed tubule-like structures, which showed no morphological difference compared with tubules induced by HGF alone (data not shown). To compare the tubulogenic activity of HGF and follistatin in MDCK cells cultured in collagen gel, semiquantitative analysis was also performed as described in the **Methods** section. As shown in Table 1, there was no significant difference between HGF and follistatin in tubulogenic activity, but their effect on the tube formation was additive. The number of branch points in tubules induced by follistatin was almost similar to that of HGF (Fig. 5E).

Induction of tubule formation by transfection of truncated type II activin receptor

These results prompted us to determine whether follistatin exerts tubulogenic activity by blocking the effect of endogenous activin A. To this end, we attempted to introduce cDNA encoding a tARII by introducing the adenovirus vector into the spherical cysts in collagen gel. This truncated receptor, which lacks the intracellular serine/threonine kinase domain, inhibits activin signaling in a dominantly negative fashion [20]. To determine the efficacy of transfection using the adenovirus vector, we transfected spherical cysts in collagen gel with AdexLacZ. *LacZ* gene expression was examined by histochemical staining of β-galactosidase at the indicated times. When transfected with AdexLacZ, almost all cysts exhibited β-galactosidase activity. However, no such activity was seen in untreated cysts (Fig. 7). The morphological appearance of the treated cysts was indistinguishable from untreated cysts. The β-galactosidase activity was detected at 24 hours and persisted for at least 6 days after infection (Fig. 8A).

The activity was dependent on the viral concentration (Fig. 8B). These results showed the stable expression of the introduced gene and confirmed successful gene transfer by the adenovirus vector in the collagen gel culture without significant cell injury.

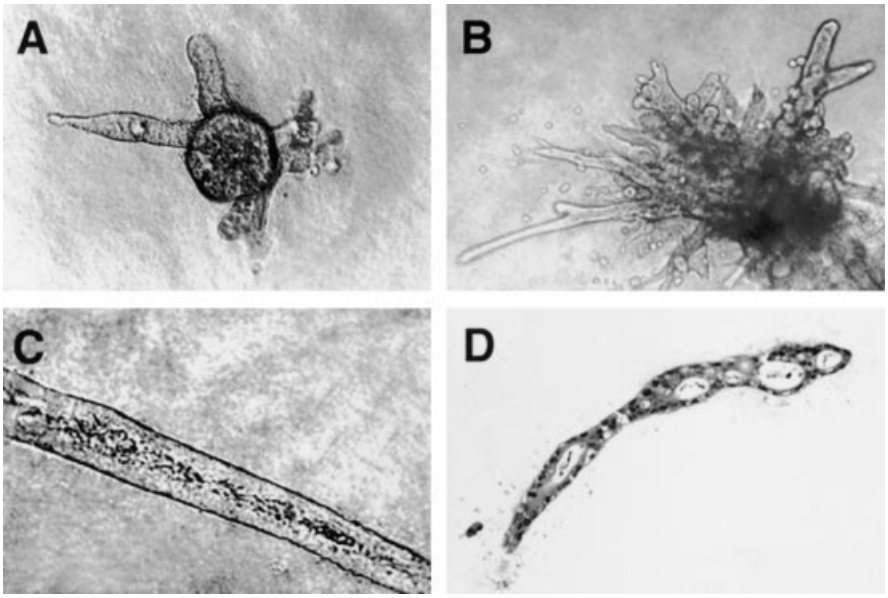


Fig. 5. Effect of follistatin on the morphology of MDCK cells cultured in collagen gel. MDCK cells were cultured in collagen gel in the presence of 10 nmol/L follistatin for the indicated time. (*A*) Six days (×200). (*B*) Nine days (×100). (*C*) Eleven days (×400). (*D*) Nine days, hematoxylin and eosin staining (×400).

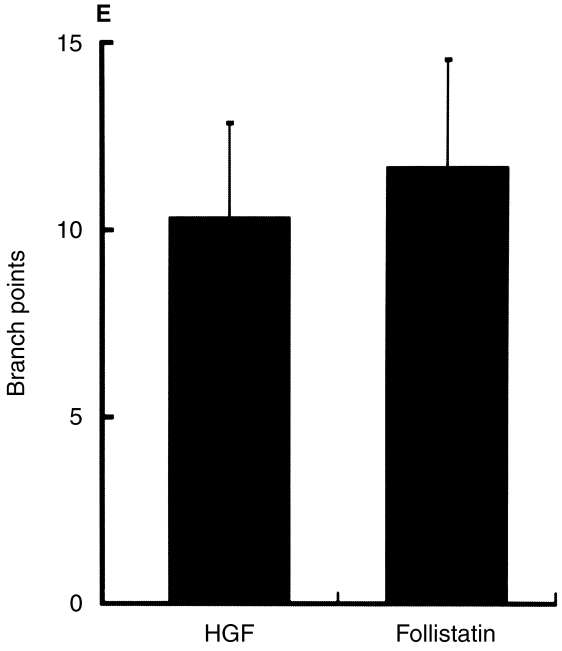


Fig. 5. (*E*) Quantitation of branch points in tubules induced by HGF or follistatin. Values are the mean ± SE of three experiments.

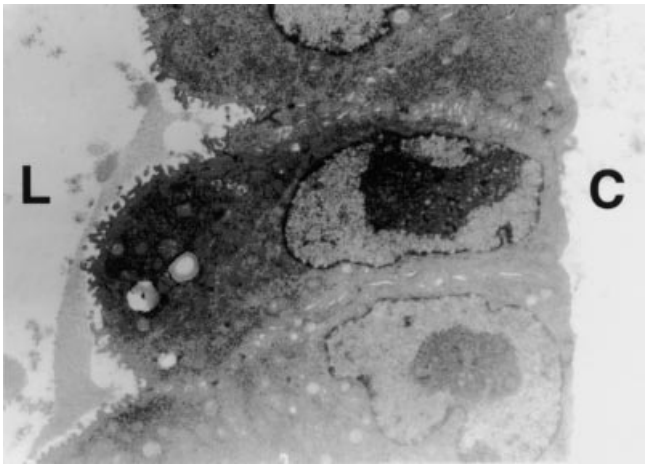


Fig. 6. Ultrastructural morphology of branching tubules formed by MDCK cells treated with follistatin. Thin section of branching tubules formed by MDCK cells cultured with follistatin for 10 days. The lumen of the tubule was delimited by well-polarized epithelial cells with apical microvilli. L, lumen; C, collagen gel (×5000).

When transfected with AdextARII, the spherical cysts in collagen gel grew to form many elongated branching tubule-like structures within 48 hours after infection (Fig. 9A). These structures had slit-like translucent spaces (Fig. 9B) and were morphologically similar to those induced by HGF or follistatin. This inductive effect was observed at a viral concentration of 4×10^6 pfu/well.

To confirm the blockade of activin signaling by transfection of the truncated activin receptor, MDCK cells transfected with AdextARII were treated with activin A. Morphological appearance of branching tubules induced by transfection with AdextARII was not affected by the addition of exogenous activin A (data not shown).

Expression of the β_A subunit and follistatin in MDCK cells cultured in collagen gel

These results suggest that the blockade of activin A action leads to formation of tubule-like structures and branching morphogenesis. In the monolayer culture, HGF reduced the expression of the activin β_A subunit (Fig. 2). If HGF also inhibits the expression of β_A subunit in

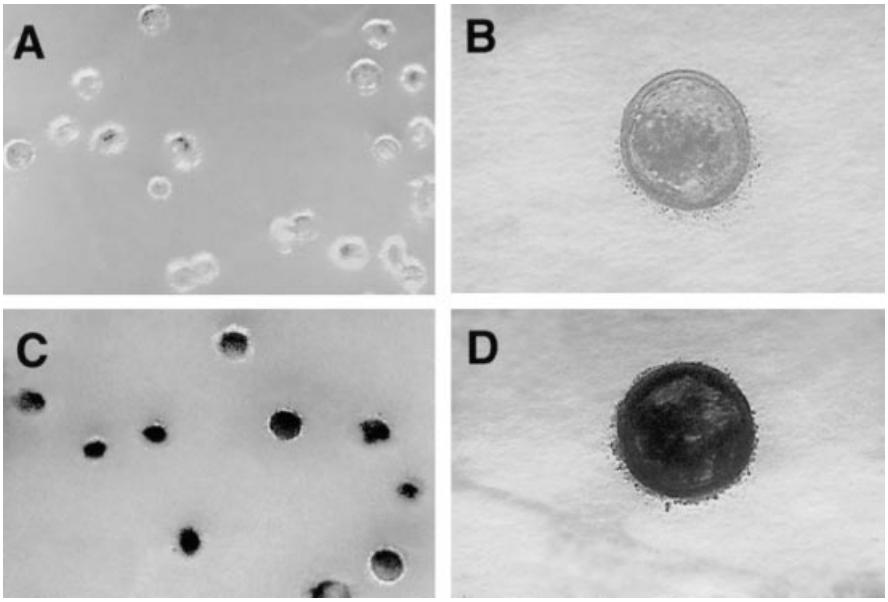


Fig. 7. Transfection of spherical cysts with AdexLacZ. Spherical cysts were transfected with (*C* and *D*) or without (*A* and *B*) AdexLacZ (4×10^6 pfu/well), and β-galactosidase activity was visualized at 24 hours after infection immunohistochemically. (*A*) None (×40). (*B*) None (×200). (*C*) Four $\times$ 10^6 pfu/well (×40). (*D*) Four x10^6 pfu/well (×200).

Table 1. Tubulogenic activities of hepatocyte growth factor (HGF) and follistatin in MDCK cells cultured in collagen gel

	Smooth cysts	Irregularly shaped cysts	Branching tubules
None	76.7 ± 5.4	21.7 ± 4.1	1.7 ± 2.0
HGF	16.7 ± 5.4	56.7 ± 2.0	26.7 ± 5.4
Follistatin	31.7 ± 14.3	40.0 ± 12.7	29.3 ± 5.4
HGF + follistatin	20.0 ± 6.1	41.7 ± 2.0	38.3 ± 4.1

Tubulogenic activities of HGF and follistatin were measured in MDCK cells cultured in collagen gel. MDCK cells were cultured with complete medium containing indicated factors for 10 days in collagen gel. Semiquantitative analysis was performed as described in the **Methods** section. Values are the mean percentage ± SE of three experiments.

MDCK cells cultured in collagen gel, this implies that HGF induces tubulogenesis and branching morphogenesis at least partly by blocking the action of activin A. To test this possibility, we measured the mRNA expression of the β_A subunit in MDCK cells cultured in collagen gel. As shown in Figure 10A and B, HGF markedly reduced the steady-state expression levels of the β_A subunit of activin. In addition, the mRNA level of β_A subunit of tubule-like structures induced by HGF was lower than that of spherical cysts (Fig. 10C). We further examined mRNA expression for β_A subunit in spherical cysts and branching tubules using in situ hybridization with digoxigenin-labeled riboprobes. As shown in Figure 11A, intense hybridization signals were observed and located within cytoplasm of MDCK cells in spherical cysts. Hybridization with control sense probe showed no positive signals (Fig. 11C). In branching tubules induced by HGF, hybridization signals were apparently diminished compared with spherical cysts (Fig. 11B). HGF did not affect the mRNA levels of follistatin, an activin A antagonist (data not shown).

DISCUSSION

In the present study, we showed that follistatin induced morphological changes in MDCK cells cultured in collagen gel. Follistatin elicited formation of tubule structures and branching morphogenesis. Qualitatively, these changes are identical to those induced by HGF. Follistatin is a specific activin-binding protein and blocks the action of activins in most biological systems by preventing the binding of activin to the activin type II receptor [12]. As shown in Figures 1 and 2, activin A, a homodimer of the β_A subunit, is produced in these cells. Follistatin may induce morphological changes in MDCK cells by canceling the action of the autocrine factor activin A. This tubulogenic action of follistatin may result from the induction of other "tubulogenic" factors. A variety of growth factors including acidic fibroblast growth factor (FGF), basic FGF, TGF-β, platelet-derived growth factor, EGF, IGF-I, IGF-II, and keratinocyte growth factor (KGF) do not have any tubulogenic activity in MDCK cells [4]. OP-1/BMP-7 does induce tubulogenesis, but follistatin does not affect the expression of OP-1/BMP-7 (data not shown). It is thus likely that follistatin acts as an antagonist of activin in this model rather than producing tubulogenic factors. This notion was supported by the transfection of the gene encoding the tARII. This mutant receptor inhibits the signaling via activin receptor in a dominant-negative fashion [20]. As depicted in Figure 9, the introduction of the dominant-negative activin receptor completely reproduced the effect of follistatin, supporting the idea that follistatin induces morphological changes by blocking the action of activin A. It should be noted that follistatin also traps OP-1/BMP-7 [13], and in addition, OP-1/BMP-7 also binds to the type II activin recep-

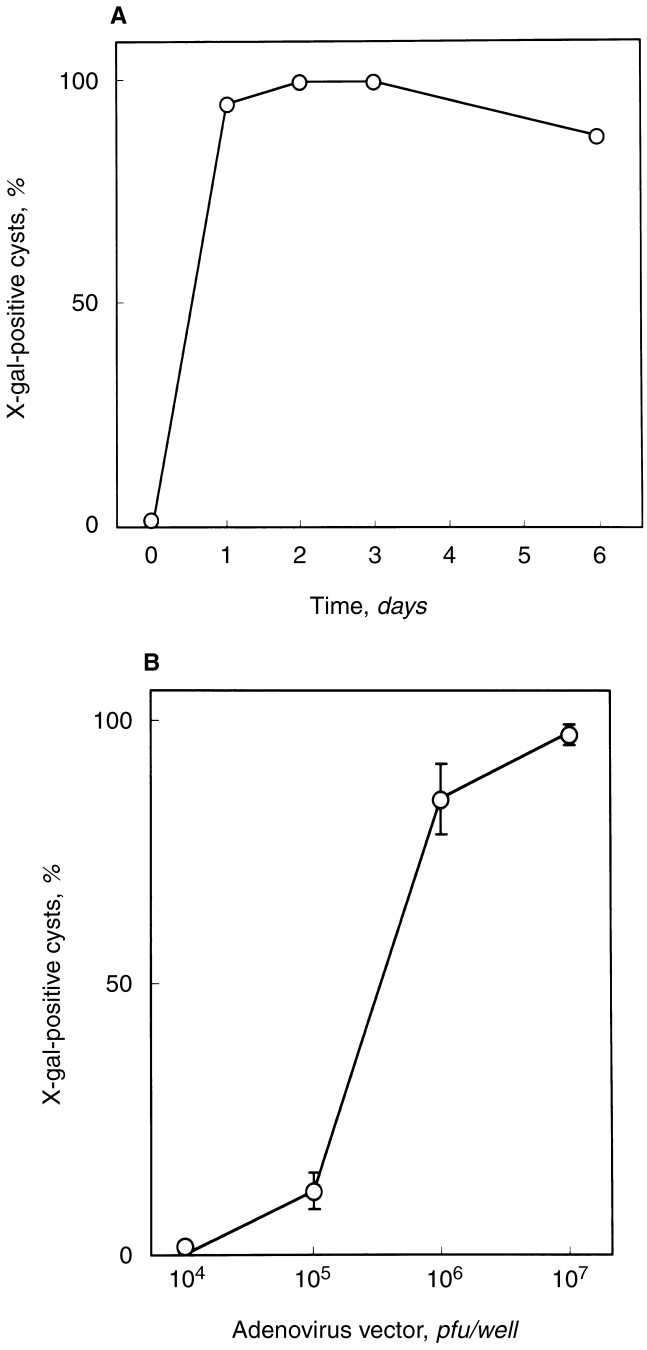


Fig. 8. Time course and dose–response relationship for the transfection of AdexLacZ. (*A*) Spherical cysts were transfected with AdexLacZ (4×10^6 pfu/well), and β-galactosidase activity was detected at the indicated time. The number of X-gal–positive cysts was counted. Values are the mean ± SE of four experiments. (*B*) Spherical cysts were incubated with various concentrations of AdexLacZ for 24 hours, and the number of X-gal–positive cysts was counted.

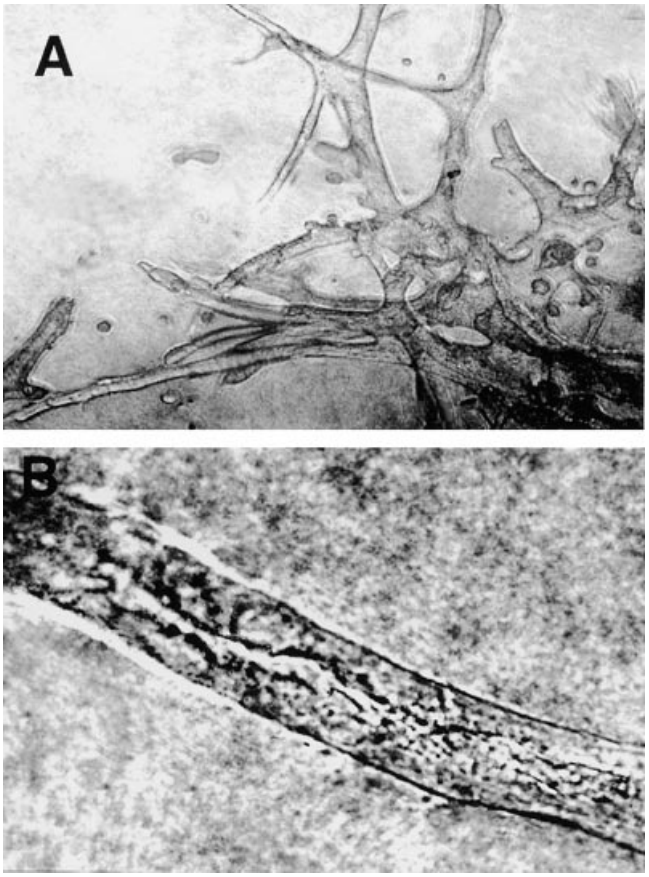


Fig. 9. Transfection of spherical cysts with AdextARII. Spherical cysts were incubated with AdextARII (4×10^6 pfu/well) for 24 hours. (*A*) ×100. (*B*) ×400.

tor [22, 23]. Therefore, it is also possible that follistatin exerts its action by blocking the action of OP-1/BMP-7. We consider this possibility unlikely for the following reason. OP-1/BMP-7 was shown to promote or inhibit branching morphogenesis depending on its concentration in mIMCD-3 renal tubular cells [24]. In MDCK cells, OP-1/BMP-7 promotes branching morphogenesis without any inhibition of tubulogenesis (A. Maeshima and I. Kojima, unpublished observation). Therefore, a blockade of OP-1/BMP-7 action would inhibit branching morphogenesis. Collectively, a blockade of the action of activin A either by the addition of follistatin or transfection with the AdextARII induces morphogenesis identical to HGF action. These observations raise an interesting possibility that MDCK cells possess an intrinsic capability to form tubules in collagen gel and that activin A synthesized in MDCK cells exerts a tonic inhibition on tubule formation. Alternatively, MDCK cells produce an autocrine factor that promotes tubulogenesis and activin A tonically inhibits its action. In any event, HGF blocks the expression of activin A and thereby reduces the tonic inhibition. It is also possible that HGF attenuates activin signaling such as down-regulation of activin receptors. Hence, HGF may induce branching tubulogenesis by reversing the tonic inhibition of activin A. In a strict sense, it is not clear at present whether HGF

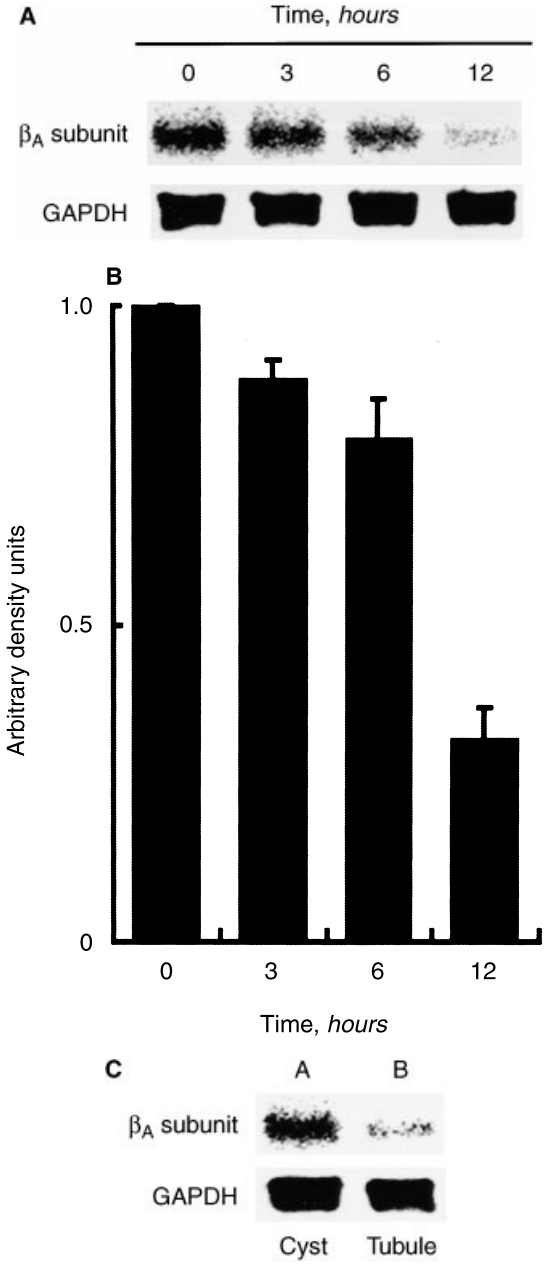


Fig. 10. Effect of HGF on the expression of mRNA for the β_A subunit and follistatin. (*A*) Spherical cysts were incubated with 250 pmmol/L HGF for the indicated time. The gel was then digested with collagenase, and the mRNA was extracted. mRNA expression for the β_A subunit and GAPDH was measured by Northern blotting. (*B*) Quantitation of β_A subunit/GAPDH mRNA in MDCK cells treated with HGF for indicated times in collagen gel. Values are the mean ± SE of three experiments and are expressed as arbitrary density units. (*C*) mRNA was extracted from spherical cysts and HGF-induced branched tubules. The expression of mRNA for β_A subunit, and GAPDH was measured.

induces branching tubulogenesis solely by reducing the tonic inhibition of activin A. In this regard, follistatin and transfection of the dominant-negative activin receptor essentially reproduce the effect of HGF. Furthermore, HGF was not capable of inducing tubulogenesis when the action of activin A was maintained by adding an exogenous ligand. Therefore, it is reasonable to conclude that inhibition of activin production accounts for most of the tubulogenic action of HGF. Interestingly, in the presence of activin A, HGF could not induce tubulogenesis, but instead, MDCK cells were scattered. Activin A blocks tubulogenesis, but in the presence of activin A, a scattering action of HGF was observed. When HGF induces tubulogenesis, this scattering action is probably suppressed because of the induction of cell adhesion. When tubulogenesis is blocked by activin A, cells then scatter since cell adhesion may not be tight.

The mechanisms involved in the HGF-induced tubulogenesis is likely to be complex, and many intracellular signaling molecules may initiate multiple signal cascades eventually leading to tubulogenesis. Elevated phosphatidylinositol (PI) 3-kinase activity induces tubulogenesis [25]. Tubulogenic action of HGF is mediated by Stat3 [26]. These signaling pathways are necessary, but not sufficient for tubulogenesis. Therefore, there may be other signaling molecules or cross-talk between a different signaling pathway.

The Smad proteins mediate signals from receptor serine/threonine kinase of activin or other members of the TGF-β superfamily. Smad is also a target of HGF signaling through receptor tyrosine kinase [27, 28]. TGF-β/Smad signaling is inhibited by the interferon-γ/Stat pathway [29]. These reports suggest that the interaction between Stat and the Smad pathway opposes the effect of activin and HGF in this model. Further study is needed to address this issue. As HGF-induced reduction of mRNA expression for β_A subunit was blocked by PI 3-kinase inhibitors (wortmannin and LY290042; unpublished observation), modulation of mRNA expression for β_A subunit though PI 3-kinase signaling may be at least one of the downstream pathways involved in HGF-induced tubulogenesis.

Extracellular proteases, protease inhibitors, extracellular matrix proteins, and integrins are likely to act as effectors and regulators of branching morphogenesis. HGF increases urokinase-type plasminogen activator (u-PA) and u-PA receptor expression in MDCK cells [30]. TGF-β altered the profile of extracellular matrix-degrading proteases and their inhibitors [31]. Our unpublished observations indicate that activin did not alter the expression of HGF or other inhibitory regulators such as TGF-β [31] and BMP-7 [24] in MDCK cells (data not shown). Therefore, activin may also modulate the expression of regulators of proteolysis by a direct mechanism.

The present results indicate that HGF causes branching tubulogenesis by modulating the activin-follistatin system rather by a direct mechanism. This implies that in addition to HGF, tubulogenesis in developing kidneys may also be modified directly by the activin-follistatin system. Since follistatin is expressed abundantly in renal

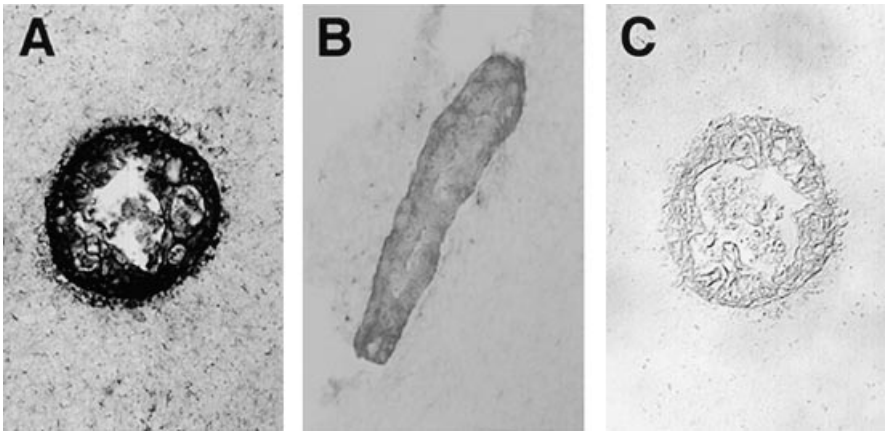


Fig. 11. Detection of β_A subunit mRNA in MDCK cells exhibiting spherical cyst and branching tubule by in situ hybridization. Cysts were incubated with (*B*) or without (*A* and *C*) 250 pmol/L HGF for 10 days, and mRNA for β_A subunit was detected by in situ hybridization. (A and B) Antisense probe. (C) Sense probe (magnification ×400).

tubules [15] and in developing kidney [18], follistatin may also regulate branching tubulogenesis in vivo. Many kinds of knockout mice that lack the activin subunit or activin receptors or follistatin have been developed [32–35]. However, abnormality of the kidney was not described in these mutant mice, most likely due to the redundancy in the ligands and receptor systems. We recently observed that in transgenic mice expressing dominant-negative activin receptor, the number of glomeruli was increased [37]. This increase in the nephron number may result from the enhanced tubular branching during development caused by the inhibition of the activin signal. Therefore, the activin-follistatin system may be critical for the development of nephrons in the kidney.

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